Pseudofeedback Inhibition of Purine Synthesis by 6-Mercaptopurine Ribonucleotide and Other Purine Analogues*

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Exogenous purines are known to suppress purine synthesis de novo in bacteria (2-4), HeLa and L cells (5), and ascites cells (6) growing in culture. High levels of purines, especially adenine, inhibit accumulation of late purine precursors in the media of bacteria with blocks in the pathway of purine synthesis (2, 3), or of formylglycinamide ribonucleotide in Ehrlich ascites cells grown in the presence of azaserine (6). These studies have implicated an early step in purine biosynthesis as the site of a feedback regulatory mechanism. Studies from this laboratory have shown that this inhibition operates on the first specific reaction of purine biosynthesis in which ribosylpyrophosphate 5-phosphate and glutamine interact to form phosphoribosylamine, and that adenosine triphosphate is the most potent of the inhibitory purine ribonucleotides (7, 8).

Several purine analogues have also been shown to affect purine synthesis de novo. Goto and Collub (9) observed that 6-mercaptopurine and 6-thioguanine inhibited accumulation of 5-formylglycinamide ribonucleotide in Ehrlich ascites cells or mouse neoplasms treated with azaserine. Simpson, Bennett, and Golden (10) and Le Page and Jones (11) observed that purine analogues inhibited accumulation of formylglycinamide ribonucleotide in Ehrlich ascites cells or mouse neoplasms treated with azaserine. Simpson, Bennett, and Golden (12) described the inhibition of incorporation of formate-14C into purines in Ehrlich ascites cells under conditions in which the terminal steps of purine synthesis were not inhibited. All of these studies suggested that these purine analogues might also be inhibiting purine synthesis by acting on an early reaction of the sequence. This paper presents evidence that the ribonucleotide derivatives of 6-mercaptopurine, 6-thioguanine, 8-azaguanine, and 4-hydroxypyrazolo-(3,4-d)pyrimidine effect a pseudofeedback inhibition of glutamine ribosylpyrophosphate 5-phosphate amidotransferase, mimicking the inhibitory action of natural purine ribonucleotides on this enzyme.

EXPERIMENTAL PROCEDURE

Materials—6-Mercaptopurine: H2O, 6-mercaptopurine ribonucleoside, 6-thioguanine (California Corporation for Biochemical Research), 8-azaguanine (Nutritional Biochemicals Corporation) adenine 5'-phosphate, ribosylpyrophosphate 5-phosphate, the 3-acetylpuridine analogue of NAD (Pabst Laboratories), L-glutamine (Mann Research Laboratories), glutamic dehydrogenase in ammonium sulfate or 50% glycerol (Sigma Chemical Company), and xanthine oxidase (Worthington Biochemical Corporation) were commercial preparations. 4-Hydroxypyrazolo-(3,4-d)pyrimidine (Burroughs Wellcome Company) was a generous gift of Dr. Gertrude Elion and Dr. George Hitchings. Solutions of PP-ribose-P were assayed according to Kornberg, Lieberman, and Simms (13).

Partial Purification of Enzymes—Inosinic-guanosine acid pyrophosphorylase was prepared from pigeon liver by a modification of the procedure of Lukens and Herrington (14) when a preparation free of contaminating xanthine oxidase activity was desired, and from hog liver according to Way and Parke (15) when a preparation free of contaminating guanase activity was desired. Pigeon liver was homogenized in 2 volumes of phosphate-KCl-MgCl2 bicarbonate buffer (16), and the supernatant fraction was obtained by centrifugation at 100,000 X g for 45 minutes. The protein fraction precipitating between 55 and 80% of saturation after addition of saturated ammonium sulfate solution, pH 7.4, was redissolved in 0.1 M phosphate, pH 7.4, and dialyzed for 3 hours against 300 volumes of 0.003 M phosphate buffer, pH 7.4. It was then placed in a glass tube in a water bath at 75° for 5 minutes following which the tube was immediately cooled in an ice slurry. Insoluble protein was removed by centrifugation at 15,000 X g for 10 minutes. An extract of hog liver acetone powder was prepared, dialyzed, and lyophilized according to Kornberg, Lieberman, and Simms (13). Both pyrophosphorylases were assayed by following the disappearance of hypoxanthine. The assay mixture contained: hypoxanthine, 0.33 μmole; P1'-ribose-P', 1.2 μmoles; MgCl2, 3.2 μmoles; potassium phosphate buffer, pH 7.4, 75 μmoles; and enzyme solution in 3 ml. After incubation at 37° for 15 minutes the pyrophosphorylase was inactivated by placing the tube in a boiling water bath for 3 minutes. The residual hypoxanthine was determined with xanthine oxidase (17) and compared with a zero time sample in which the pyrophosphorylase was heat-inactivated before addition of hypoxanthine and P1'-ribose-P. One unit was defined as the amount of enzyme which catalyzes the release of 1 μmole of hypoxanthine in 1 hour.

1 The abbreviations used are: PP-ribose-P, ribosylpyrophosphate 5-phosphate; 6-thioGMP, 6-thioguanosine 5'-phosphate; and 8-azaGMP, 8-azaguanosine 5'-phosphate.

2 We are indebted to Dr. D. S. Newcombe for assistance in adapting the procedure of Lukens and Herrington to pigeon liver.
defined as equivalent to the disappearance of 0.1 µmole of hypoxanthine in 15 minutes. Protein was measured from the absorbances at 260 and 280 nm according to Warburg and Christian (18).

Glutamine PP-ribose-P amidotransferase was purified from pigeon liver according to the procedure of Wyngaarden and Ashton (7), to which was added a final step of chromatography on DEAE-cellulose. This modified procedure yields an enzyme insensitive to ATP but sensitive to AMP. The AMP inhibition is competitive against PP-ribose-P with a $K_i$ value of $1 \times 10^{-3}$ M. Preparations used in these studies were obtained from DEAE-cellulose columns and had specific activities of 2500 to 3200 units per mg of protein.

**Biosynthesis of Purine Analogue Ribonucleotides—6-Mercaptopurine ribonucleotide was prepared biosynthetically from 6-mercaptopurine and PP-ribose-P, essentially according to Lukens and Herrington (14). 6-Mercaptopurine, 150 µmoles; PP-ribose-P, 150 µmoles; MgCl$_2$, 320 µmoles; Tris buffer, pH 8.0, 1000 µmoles; and inosinic acid pyrophosphorylase (400 units) from pigeon liver in a total volume of 250 ml were incubated at 37° for 30 minutes. Disappearance of 6-mercaptopurine was followed with xanthine oxidase according to Silberman and Wyngaarden (19). The ribonucleotide derivative of 4-hydroxy-pyrazolo-(3,4-d)pyrimidine was prepared as follows: 100 µmoles of analogue base, 55 µmoles of PP-ribose-P, 180 µmoles of MgCl$_2$, 1000 µmoles of Tris-HCl buffer, pH 7.0, and 100 mg of lyophilized extract of hog liver acetone powder were incubated in a total volume of 14 ml for 2 hours at 37°.

6-ThioGMP and 8-azaGMP were synthesized from base and PP-ribose-P in the presence of guanylic acid pyrophosphorylase from hog liver according to Way and Parks (15). In each case 80 µmoles of the guanine analogue, 80 µmoles of PP-ribose-P, 800 µmoles of MgCl$_2$, and 8000 µmoles of Tris buffer, pH 8.0, were incubated with 70 mg of enzyme at 37° for 40 minutes. Isolation of the various ribonucleotides followed the procedure of Way and Parks (19). The ultraviolet spectral properties and chromatographic mobilities of each of the analogue ribonucleotides corresponded well with published data (15). Concentrations were estimated from absorbance values and known extinction coefficients (15, 20).

**RESULTS**

**Inhibition of Glutamine PP-ribose-P Amidotransferase by Purine Analogue Ribonucleotides—**When the analogue ribonucleotides were added to the amidotransferase in the standard assay (7), significant inhibition was observed with each.4 This inhibition was progressive with increasing concentrations of analogue ribonucleotides as shown for 6-mercaptopurine ribonucleotide in Fig. 1, and for 4-hydroxy-pyrazolo-(3,4-d)pyrimidine ribonucleotide in Table I. No inhibition was observed with any of the free bases, nor with 6-mercaptopurine ribonucleoside.


4 The effects of the analogue ribonucleotides on glutamic acid dehydrogenase were also studied. Only the 6-mercaptopurine derivative was inhibitory, and at the concentrations employed in the studies on amidotransferase, the inhibition was approximately 12%. A large excess of glutamic acid dehydrogenase was employed in the assay of amidotransferase and since the inhibitions of the coupled reaction were greater than 90% in the studies described in this paper, the effect on glutamic acid dehydrogenase was considered noncontributory.

![Figure 1](http://example.com/image1.png)

**Fig. 1. Inhibition of glutamine PP-ribose-P amidotransferase by 6-mercaptopurine ribonucleotide at various levels of inhibitor.**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>A.O.D. at 311 ma</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>0.064</td>
<td>0</td>
</tr>
<tr>
<td>AMP</td>
<td>$2 \times 10^{-4}$</td>
<td>0.061</td>
<td>5</td>
</tr>
<tr>
<td>IMP</td>
<td>$2 \times 10^{-4}$</td>
<td>0.061</td>
<td>5</td>
</tr>
<tr>
<td>4-Hydroxy-pyrazolo-(3,4-d)pyrimidine ribonucleotide</td>
<td>$2 \times 10^{-4}$</td>
<td>0.054</td>
<td>16</td>
</tr>
<tr>
<td>4-Hydroxy-pyrazolo-(3,4-d)pyrimidine ribonucleotide</td>
<td>$1 \times 10^{-3}$</td>
<td>0.018</td>
<td>72</td>
</tr>
</tbody>
</table>

![Figure 2](http://example.com/image2.png)

**Fig. 2. Inhibitions of glutamine PP-ribose-P amidotransferase by 6-mercaptopurine ribonucleotide, 6-thioGMP, and 8-azaGMP as functions of the concentration of PP-ribose-P.** The inhibitions are competitive with respect to PP-ribose-P. Concentrations of inhibitors were as follows: 6-mercaptopurine ribonucleotide, $1 \times 10^{-4}$ M; 6-thioGMP, $2.9 \times 10^{-4}$ M; 8-azaGMP, $1.5 \times 10^{-4}$ M.
The data on inhibition of amidotransferase by 4-hydroxypprazolo-(3,4-d)pyrimidine ribonucleotides are shown in Table I. The enzyme-inhibitor dissociation constants\(^6\) are shown in Table II.

The inhibitors caused decreases in maximal velocities and increases in apparent \(K_m\) values of glutamine, an indication of a mixed competitive-noncompetitive inhibition with respect to this substrate.

**Cooperative Inhibition**—The inhibitory effects of AMP and 6-mercaptopurine ribonucleotide were studied singly and in combination. Table III shows the velocity data at two different concentrations of each inhibitor. The inhibitory effects of AMP and 6-mercaptopurine ribonucleotide in combination are approximately additive.

**DISCUSSION**

The inhibitions of glutamine phosphoribosylpyrophosphate amidotransferase by the analogue ribonucleotides appear to be qualitatively similar to those caused by natural purine ribonucleotides. Studies of both groups of purine compounds on synthesis de novo indicate an inhibition of an early step of the sequence (2-12). The inhibition of the amidotransferase by adenyl ribonucleotides constitutes an example of end product or negative feedback inhibition (7,8). That by the analogue ribonucleotides may therefore be viewed as a pseudofeedback inhibition. The enzymology of inhibition of the amidotransferase by purine ribonucleotides will be the topic of a separate communication.\(^5\)

There are indications that pseudofeedback inhibition by purine analogue ribonucleotides may be an important effect in vivo. The azaserine-induced accumulation of formylglycinemic ribonucleotide in Ehrlich ascites cells is inhibited by 6-mercaptopurine and less strongly by 6-thioguanine (10,11). In epithelial cells in culture this accumulation is inhibited by 6-mercaptopurine and 6-thioguanine only in strains having inosinic-guanine pyrophosphorylase activity and therefore able to convert the base analogue to its respective ribonucleotide.\(^6\) These results agree well with expectations derived from the present study.

The results of the use of 4-hydroxypprazolo-(3,4-d)pyrimidine in man also indicate the probable operation of a pseudofeedback inhibition on purine synthesis de novo. This base is a potent inhibitor of xanthine oxidase (22), and its administration results in striking reductions in levels of uric acid in serum and urine (23). However, the decrements of urinary urate excretion are accompanied by less than proportionate increments in urinary oxyurine (hypoxanthine plus xanthine) excretion, a strong suggestion of a second effect on purine synthesis de novo (23). This additional effect could well be the inhibition of amidotransferase by the ribonucleotide derivative.

Other examples of pseudofeedback inhibition include those of fluorocytidine, fluoroorotic acid, and azauridine on pyrimidine synthesis (24-26), of 6-fluorotryptophan, 4-methyltryptophan, 5-methyltryptophan, and azatryptophan (27-29) on tryptophan synthesis (24-26), of 6-fluorotryptophan, 4-methyltryptophan, and azatryptophan (27-29) on tryptophan synthesis, of 2-thiazolealanine on histidine synthesis (30).

\(^1\) These \(K_i\) values should be compared with \(K_i\) values of 1 or 2 \(\times 10^{-5}\) M for AMP with these same preparations of amidotransferase. Since there is evidence\(^6\) that the less highly purified enzyme is more sensitive to inhibition by AMP as well as by other natural purine ribonucleotides, the \(K_i\) values of the analogue ribonucleotides in vivo may be appreciably lower than recorded here.

\(^5\) R. W. Brockman, personal communication.

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**Mechanism of Inhibition**—The inhibitions were examined as functions of substrate concentrations and the data plotted according to the method of Lineweaver and Burk (21). The kinetic data for three of the analogue ribonucleotides are presented in Figs. 2 and 3. The interactions of ribonucleotides of 6-mercaptopurine, 6-thioguanine, and 8-azaguanine with the enzyme were competitive with respect to PP ribose-P. The inhibitions of glutamine phosphoribosylpyrophosphate amidotransferase by the analogue ribonucleotides appear to be qualitatively similar to those caused by natural purine ribonucleotides.
Resistance of certain bacterial strains to growth inhibition by 5-methyltryptophan or by 2-thiazolealanine has been attributed to mutational changes in the regulated enzyme rendering it insensitive to pseudofeedback control (29, 30). Since avian glutamine PP-ribose-P amidotransferase may be desensitized to feedback inhibitors in vitro, it will be important to consider mutational loss of pseudofeedback inhibition among the potential causes of relative resistance to purine analogue inhibitors in vivo.

The inhibition of amidotransferase by 6-mercaptopurine ribonucleotide thus adds another site of action to those already known for this multifunctional inhibitor, which include inhibitions of xanthine oxidase (19), inosine phosphoribulose (31), IMP pyrophosphorylase (32), IMP dehydrogenase (33), succinoacetylcyd acid synthetase (20, 33), adenylosuccinase (20, 33), nicotinamide mononucleotide adenylyltransferase (34), and IMP pyrophosphorylase (32), IMP dehydrogenase (33), suc-

\( 5\)-phosphate amidotransferase, the first enzyme of purine biosynthesis. Guanine, 8-azaguanine, and 4-hydroxypyrazolo-(3,4-d)pyrimidine are inhibitors of avian glutamine ribosylpyrophosphate 5-phosphate amidotransferase, the first enzyme of purine biosynthesis.

2. The inhibitions are kinetically competitive against ribo-

pyrophosphate 5-phosphate, mixed competitive-noncompeti-

tive against glutamine.

3. Evidence is presented that pseudofeedback inhibition by purine analogue ribonucleotides may be an important aspect of their biological activity in vivo.

SUMMARY

1. The ribonucleotide derivatives of 6-mercaptopurine, 6-thioguanine, 8-azaguanine, and 4-hydroxypyrazolo-(3,4-d)pyrimidine are inhibitors of avian glutamine ribosylpyrophosphate 5-phosphate amidotransferase, the first enzyme of purine biosynthesis.

2. The inhibitors are non-competitive against ribo-

pyrophosphate 5-phosphate, mixed competitive noncompeti-

tive against glutamine.

3. Evidence is presented that pseudofeedback inhibition by purine analogue ribonucleotides may be an important aspect of their biological activity in vivo.

REFERENCES


18. Warrburg, O., and Christian, W., Biochim. Z., 310, 384 (1941-1942).


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